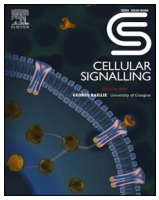
Cellular Signalling 107 (2023) 110684

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig



IL-1β stimulates a novel, IKKα -dependent, NIK -independent activation of non-canonical NFκB signalling

Kathryn McIntosh [a,*], Yousif H. Khalaf [a], Rachel Craig [a], Christopher West [a], Ashley McCulloch [a], Ajay Waghmare [a], Christopher Lawson [a], Edmond Y.W. Chan [b], Simon Mackay [a], Andrew Paul [a], Robin Plevin [a,*]

[a] Strathclyde Institute for Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow, G4 0RE Scotland, UK
[b] Department of Biomedical and Molecular sciences, Queens University, Botterell Hall, Room 563, 18 Stuart Street, Kingston, ON K7L 3N6, Canada

ARTICLE INFO

Keywords:
Nuclear factor kappa B
Non-canonical
IKKα
Lymphotoxin
Interleukin-1β

ABSTRACT

In this study, we examined the activation of non-canonical nuclear factor Kappa B (NFκB) signalling in U2OS cells, a cellular metastatic bone cancer model. Whilst Lymphotoxin α1β2 ($LT\alpha_1\beta_2$) stimulated the expected slow, delayed, sustained activation of serine 866/870 p100 phosphorylation and increased cellular expression of p52 NFκB, we found that canonical agonists, Interleukin-1β (IL-1β) and also Tumour necrosis factor-α (TNFα) generated a rapid transient increase in pp100, which was maximal by 15–30 min. This rapid phosphorylation was also observed in other cells types, such as DU145 and HCAECs suggesting the phenomenon is universal. IKKα deletion using CRISPR/Cas9 revealed an IKKα-dependent mechanism for serine 866/870 and additionally serine 872 p100 phosphorylation for both IL-1β and $LT\alpha_1\beta_2$. In contrast, knockdown of IKKβ using siRNA or pharmacological inhibition of IKKβ activity was without effect on p100 phosphorylation. Pre-incubation of cells with the NFκB inducing-kinase (NIK) inhibitor, CW15337, had no effect on IL-1β induced phosphorylation of p100 however, the response to $LT\alpha_1\beta_2$ was virtually abolished. Surprisingly IL-1β also stimulated p52 nuclear translocation as early as 60 min, this response and the concomitant p65 translocation was partially reduced by IKKα deletion. Furthermore, p52 nuclear translocation was unaffected by CW15337. In contrast, the response to $LT\alpha_1\beta_2$ was essentially abolished by both IKKα deletion and CW15337. Taken together, these finding reveal novel forms of NFκB non-canonical signalling stimulated by ligands that activate the canonical NFκB pathway strongly such as IL-1β.

1. Introduction

The nuclear factor Kappa B cascade is a major multi-component transcription factor pathway which plays a key role in both normal physiology and diseases including arthritis, cancer, cardiovascular disease and CNS disorders [1]. The cascade has two major components; the classical or canonical pathway and the non-canonical pathway [2]. Both these pathways are believed to be regulated through a multi-kinase complex known as inhibitory kappa B kinase (IKK) comprising of the active intermediates, IKKα, IKKβ and additionally IKKγ/NEMO.

Whilst the NFκB pathway is strongly activated by multiple stimuli including the cytokines IL-1β and TNFα, the non-canonical pathway (see review by [3]) is activated by a very limited group of ligands usually members of the TNFα superfamily such as CD40, lymphotoxin-β ($LT\alpha_1\beta_2$) and LIGHT [4]. Following receptor activation, stabilization and activation of NFκB inducing-kinase (NIK) promotes the activation of IKKα which in turn promotes the phosphorylation and degradation of a large IκB, p100 NFκB2, which liberates the NFκB isoform, p52 which translocates to the nucleus to regulate gene transcription. NIK is also critically involved in the degradation/processing of p100 by inducing the binding of p100 to the beta-transducin repeats-containing proteins (β-TrCP) E3 ubiquitin ligase [5–7]. However, in resting cells the levels of NIK itself are also very low since NIK is continually targeted to the proteasome via TRAF3 [8] and TRAF2/cIAP1/2 [9,10]. Thus, the activation of this pathway by cognate ligands is characteristically slow with a significant delay, and dependent on the release of NIK from proteasomal degradation.

In bone the non-canonical NFκB pathway is linked to

* Corresponding authors.
E-mail addresses: kathryn.a.mcintosh@strath.ac.uk (K. McIntosh), r.plevin@strath.ac.uk (R. Plevin).

https://doi.org/10.1016/j.cellsig.2023.110684
Received 4 October 2022; Received in revised form 13 April 2023; Accepted 17 April 2023
Available online 18 April 2023

osteoclastogenesis and differentiation through p52 and RelB subunits [11,12] and the development of arthritis [13]. However relatively little information is available linking non-canonical NFκB signalling to the development of osteosarcoma. The canonical pathway ligand IL-1β has previously been shown to drive bone metastasis of breast and prostate cancer [14,15] and more recently to mediate PD1 expression in osteosarcoma cell lines [16] however, few papers focus on IL-1β function in bone cancer. However, IL-1β strongly stimulates bone reabsorption [17,18] and a previous paper has implicated a role for p52 [19] suggesting the potential of coupling of the IL-1β receptor to the non-canonical NFκB pathway. Thus, understanding better how non-canonical NFκB signalling is propagated in bone cancer cells in response to different ligands and its potential for pharmacological inhibition is important in the development of new therapies [20].

Therefore, in this study we examined the activation of the non-canonical NFκB pathway in the osteosarcoma cell line U2OS. To our surprise, we found that IL-1β stimulated a rapid activation of phosphorylation of p100, a key initial marker of the non-canonical pathway, relative to slow activation induced by lymphotoxin-β ($LT\alpha_1\beta_2$). Using CRISPR/Cas9 IKKα deletion and pharmacological approaches, we found activation by IL-1β to be regulated by IKKα but independent of NIK. IL-1β also stimulated early activation of p52 NFκB nuclear translocation, which was partially IKKα sensitive and NIK independent. These findings indicate a novel signalling modality not fully explored for activation of the non-canonical NFκB pathway.

2. Materials and methods

2.1. Reagents

All reagents were from Sigma-Merck (Poole UK), unless otherwise stated. Antibodies were purchased as follows: pp100 (serine 866/870), p100/p52, IKKα, IKKβ, pp65 NFκB, p65 NFκB, IκBα, nucleolin, RelB and GAPDH from Cell Signalling technology (Europe); pp100 (serine 872) from Sigma, FITC anti-Rabbit and HRP-conjugated secondary antibody from Jackson Immuno Research laboratories Inc. (West Grove, PA, USA) [21]. The NIK inhibitor, CW15337, was synthesised and characterised as outlined previously [22]. The IKK2-XI inhibitor was from Calbiochem (CAS 354810–80-3) and purchased through Sigma-Merck (Poole UK). LentiCas9-Blast, LentiCas9n (D10A)-Blast, psPAX2 and pCMV-VSV-G plasmids were kind gifts from Feng Zhang and purchased from Addgene (Addgene plasmid # 52962; http://n2t.net/addgene:52962; RRID: Addgene_52,962) [23]. The agonists used for stimulation were purchased as follows, both Human recombinant TNFα and IL-1β from Insight Biotechnology (Wembley, UK) and Human recombinant Lymphotoxin α1β2 from R&D systems (McKinley Place NE, Minneapolis).

2.2. Cell culture

U2OS cells were purchased from ECACC (cat: 92022711) and cultured routinely with McCoy's 5A modified Medium (Life Technologies, Paisley, UK) supplemented with 10% (*v*/v) FCS, 10 units/ml Penicillin/Streptomycin, and 10 mg/ml L-Glutamine (Life Technologies); medium was changed every 2 days thereafter until cells became confluent. DU145 cells were purchased from ATCC (cat:HTB-81) and cultured routinely with Dulbecco's modified eagle medium (Life Technologies, Paisley, UK) supplemented with 10% (*v*/v) FCS, 10 units/ml Penicillin/Streptomycin, and 10 mg/ml L-Glutamine (Life Technologies); medium was changed every 2 days thereafter until cells became confluent. Cells were incubated at 37 °C in humidified air with 5% CO_2 and rendered quiescent by serum deprivation for 24 h prior to stimulation in serum-free medium. Human coronary artery endothelial cells (HCAECs) (Promocell, Heidelberg, Germany) were grown in endothelial cell growth medium MV2 supplemented with 5% foetal calf serum, 5 ng/ml hEGF, 10 ng/ml hBFGF, 20 ng/ml insulin-like growth factor, 0.5 ng/ml hVEGF, 1 μg/ml ascorbic acid and 0.2 μg/ml hydrocortisone (each aliquot of cryopreserved HCAECs were from a single donor). All experiments were performed between passages 2 and 6. These cells could not be serum starved, so all stimulations were in full growth media.

2.3. Cell stimulations

Cells were plated in 6 or 12-well plates and upon reaching confluence were rendered quiescent in serum-free media. Cells were stimulated with IL-1β or TNFα at a concentration of 5 ng/ml for increasing time-periods, stimulation with Lymphotoxin α1β2 was using 20 ng/ml for increasing time-periods. Post stimulation, media was aspirated and the cell monolayer washed with sterile PBS. Samples were then prepared as described for Western blotting of whole cell lysates, and or extraction of nuclear extracts.

2.4. CRISPR deletion of IKKα

Guide RNA plasmids incorporating sequences for IKKα (CHUK) were obtained from Addgene (USA). A set of three different sgRNA designs targeting different locations of IKKα were used and from these one plasmid was selected which demonstrated complete targeting of IKKα protein expression, this was CHUK gRNA (BRDN0001149372) and was a gift from John Doench & David Root (Addgene plasmid # 77033; http://n2t.net/addgene:77033; RRID:Addgene_77033) [24]. The gRNA sequences for IKKα were packaged in lentiCas9-Blast viral plasmids. U2OS cells were infected with the lentiviral IKKα gRNA plasmids and stable lines generated using antibiotic resistance (puromycin).

2.5. SiRNA silencing of IKKs

U2OS cells were transfected with ON-TARGET plus siRNA (Thermo Scientific) against sequences for IKKα (Human CHUK, #J-003473–09) or ON-TARGET plus siRNA (Thermo Scientific) against sequences for IKKβ (Human IKBKB, #J-003503–13) and non-targeting sequence (NT) was used as a negative control (Non-targeting #1, #D-001810–01-20). Lipofectamine RNAiMAX (Life Technologies) diluted in Opti-MEM (Life Technologies) was used to deliver the siRNA to the cells over 72 h. After 18 h media was replaced with normal McCoy's 5A medium containing 10% (*v*/v) FBS for a further 24 h prior to rendering quiescent.

3. Western blot analysis

Whole cell lysates and nuclear extracts prepared from U2OS were assessed for multiple phosphorylated and total proteins using Western blotting as described previously [25].

3.1. Nuclear extract preparation

Quiescent confluent U2OS cells grown on 6-well plates were stimulated as indicated in the relevant figures. Following washing in ice cold PBS, cells were scraped into PBS and centrifuged at 13,000*g* for 1 min at 4 °C. Pellets were re-suspended in 200 μl buffer 1 (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 10 mg/ml pepstatin pH 7.9) and incubated on ice for 15 min. After addition of 25 μl 10% (*w*/*v*) NP-40, samples were vortexed and centrifuged at 13,000*g* for 2 min. Pellets were re-suspended in 50 μl buffer 2 (20 mM HEPES, 25% (*v*/v) Glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 1× aprotinin, 1× leupeptin, 1× pepstatin pH 7.9) and vortexed briefly before shaking (IkA-Vibrax-vxr) at 4 °C for 15 min. Samples were then sonicated (Ultrawave U50) on ice for 1 min and centrifuged at 13,000*g* for 30 min. Samples were equilibrated for protein content before Western blotting.

3.2. Data analysis

Each figure represents one of at least three separate quantifiable

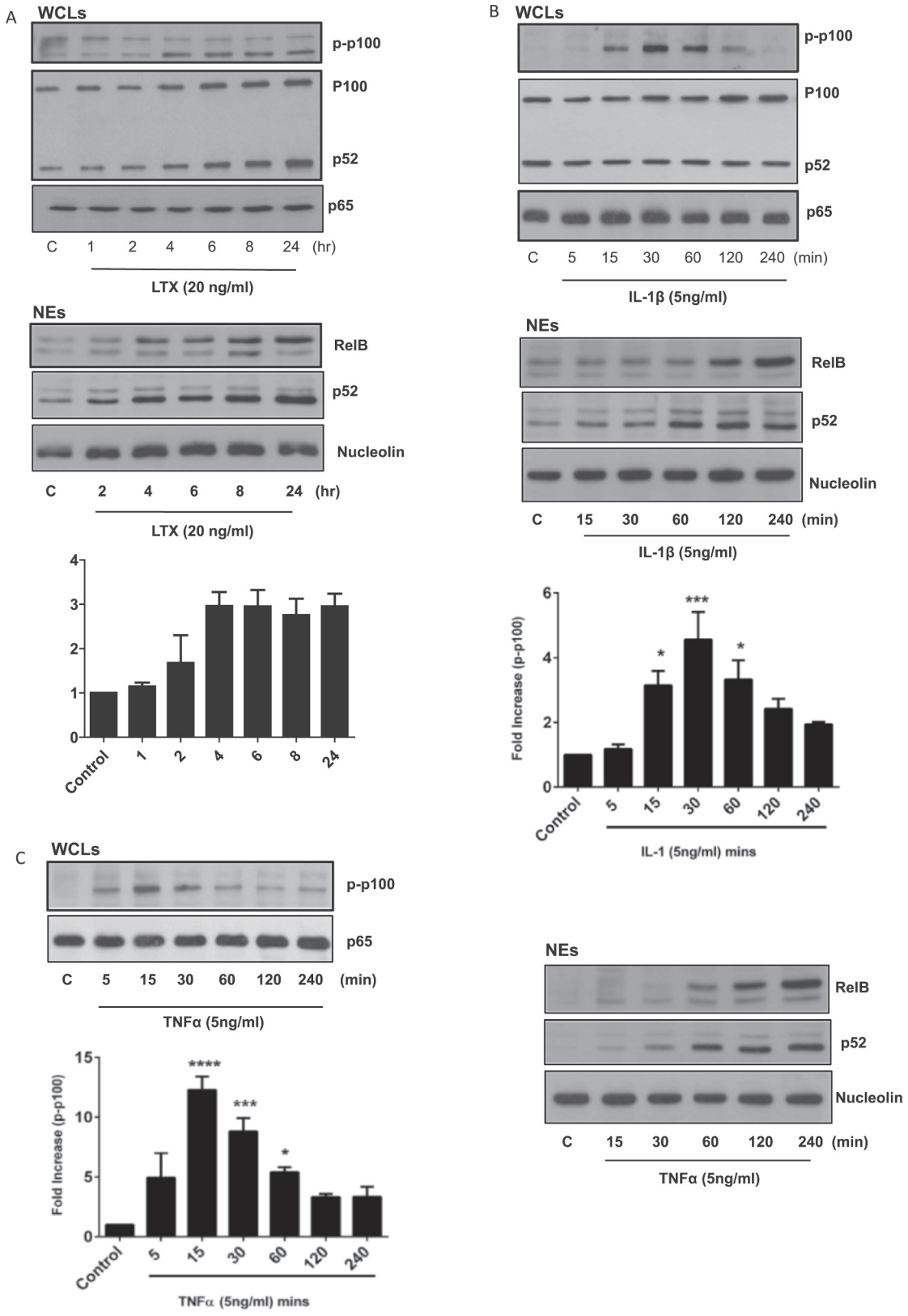


Fig. 1. Early activation of p100 phosphorylation by IL-1β and TNFα in U2OS cells U2OS cells were incubated with $LT\alpha_1\beta_2$ (Panel A), IL-1β (Panel B) or TNFα (Panel C) for the indicated time points. Whole cell lysates or nuclear extracts were assessed using Western blotting for anti-pp100 or anti-p100/p52, RelB and anti-p65/nucleolin as a loading control. Blots were semi-quantified using densitometry; results are representative of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ relative to baseline control.

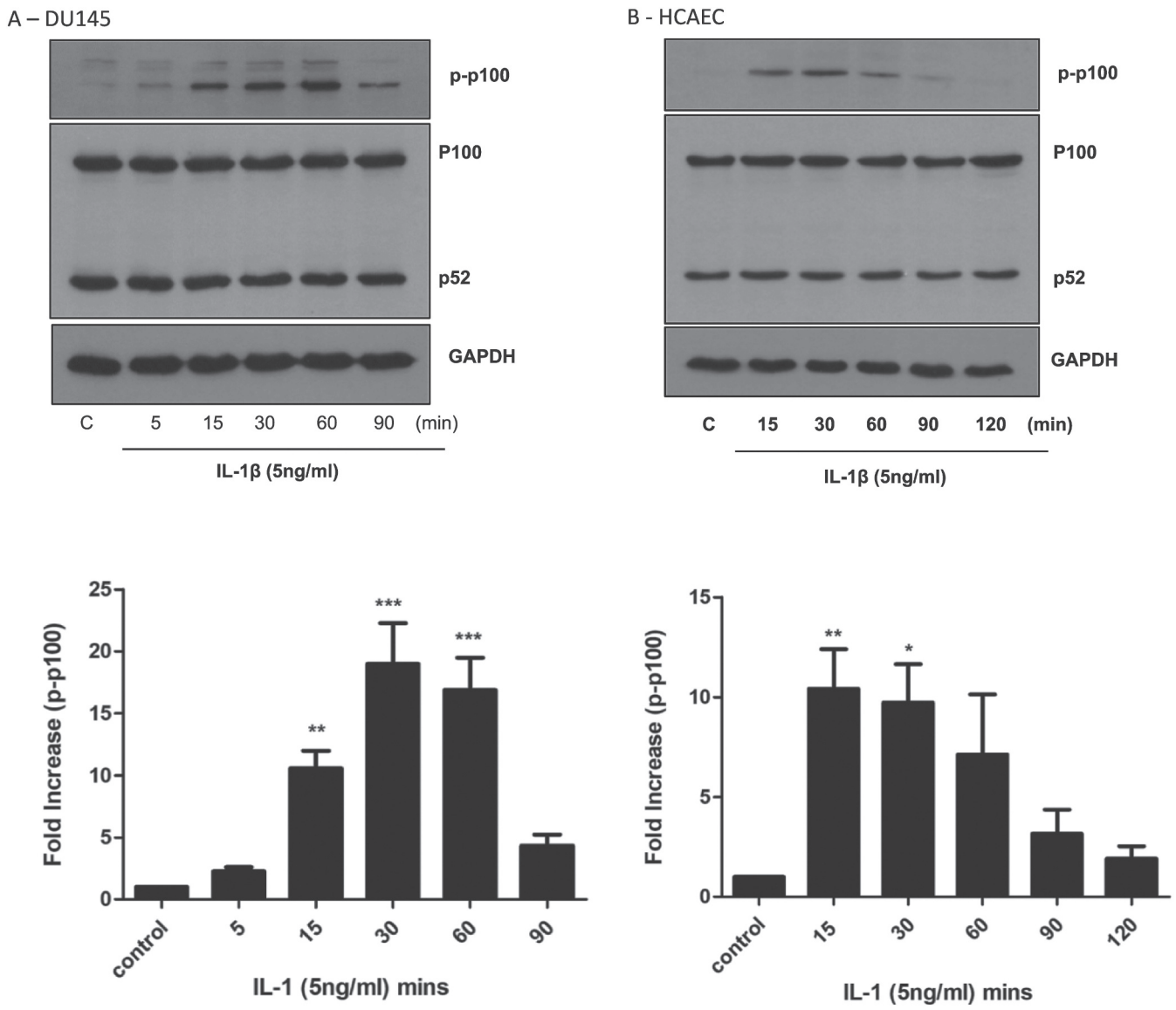


Fig. 2. Early activation of p100 phosphorylation by IL-1β in DU145 cells and Human coronary artery endothelial cells.
DU145 cells (panel A) or HCAECs (panel B) ells were incubated with IL-1β(5 ng/ml) for the indicated time points. Whole cell lysates were assessed using Western blotting for anti-pp100, anti-p100/52 and anti GAPDH as a loading control. Blots were semi-quantified using densitometry; results are representative of at least four independent experiments. *P < 0.05, **$P < 0.01$, ***$P < 0.001$, relative to baseline control.

experiments. Western blots were scanned on an Epson perfection 1640SU scanner using Adobe Photoshop 5.0.2 software. For gels, densitometry measurement was performed using the Scion Image program. Data were normalised to fold expression and expressed as mean ± s.e.m. Statistical analysis was performed by One-way ANOVA with both Dunnett's and Tukey's Post-comparison test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

4. Results

Initially we examined the kinetics of $LT\alpha_1\beta_2$ stimulated 866/870 p100 phosphorylation known to be involved in the activation of non-canonical NFκB signalling (Fig. 1). After a delay of some 2 h, $LT\alpha_1\beta_2$ stimulated a well-recognised, slow increase in pp100 which peaked at 4 h and was sustained for up to 24 h at approximately 3-fold (Fold increase: $LT\alpha_1\beta_2$, 4 h = 2.96 ± 0.32, **$P < 0.01$) of basal values (Panel A). This correlated with the cellular accumulation of p52 NFκB (p52) and over time, an increase in p100 formation. A concomitant increase in nuclear translocation of p52 was also observed (Panel A, lower blot). In contrast, we identified a far earlier kinetic profile for responses to IL-1β; the phosphorylation of p100 was rapid with onset as early as 15 min with stimulation maximal by 30 min (Fold increase: IL-1β, 30 min = 4.56 ± 0.86, ***$P < 0.001$) (Panel B). Phosphorylation levels declined and returned to basal values by 4 h. There was no overall change in cellular p52 with a slight increase in p100 expression over time. Nevertheless, there was a small increase in nuclear p52 after 15 min, which was maximum by 60 min (Fold increase: IL-1β, 60 min = 3.08 ± 0.5, **$P > 0.01$) concomitant with a sharp increase in nuclear RelB relative to basal (Fold increase: IL-1β, 240 min = 6.76 ± 0.6, ***$P > 0.001$), the delay presumably due to a prerequisite induction of RelB [26]. TNFα also stimulated this novel early activation of p100 phosphorylation, the response reaching a peak at 15 min. This again was associated with an early increase in nuclear translocation of p52 apparent within 30 min and maximum by 60 min with a delayed increase in RelB (Panel C, right-hand blots).

The ability of IL-1β to stimulate p100 phosphorylation was also

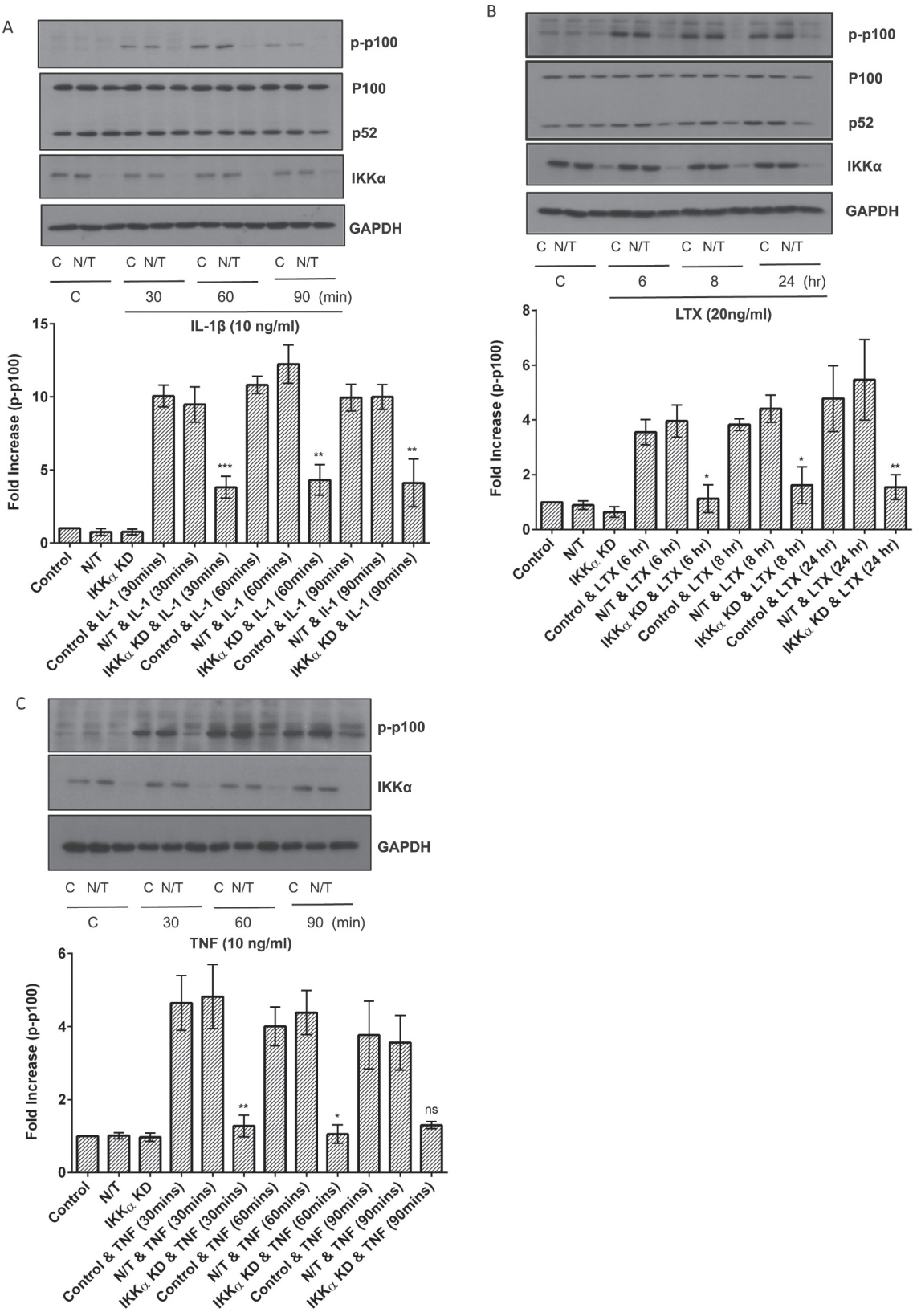


Fig. 3. CRISPR/Cas9 deletion of IKKα inhibits IL-1β, TNFα and $LT\alpha_1\beta_2$ -mediated phosphorylation of p100 in U2OS cells.
WT, Cas 9 (N/T) or IKKα CRISPR/Cas9 (IKKα-) U2OS cells were incubated with IL-1β (5 ng/ml) (Panel A), LTα1β2 (20 ng/ml) (Panel B) and TNFα (10 ng/ml) (Panel C) for the times indicated. Whole cell lysates were assessed for, p-p100, p100/p52, IKKα and GAPDH used as loading control. Blots were semi-quantified using densitometry; results are representative of at least three independent experiments. $^{*}P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with agonist-stimulated WT control.

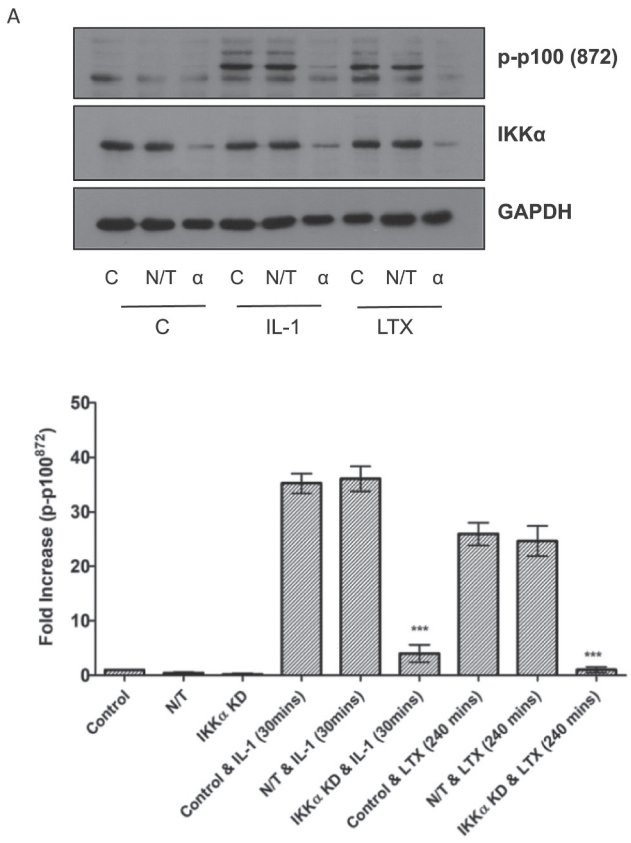


Fig. 4. IKKα deletion inhibits IL-1β and LTα$_1$β$_2$ -stimulated serine 872 phosphorylation of p100 in U2OS cells.
WT, Cas 9 (N/T) or IKKα CRISPR/Cas9 U2OS cells were incubated with IL-1β (5 ng/ml) and LTα1β2 (20 ng/ml) (Panel A) for the times indicated. Whole cell lysates were assessed for p-p100 (872), IKKα and GAPDH used as loading control. Blots were semi-quantified using densitometry; results are representative of at least three independent experiments. ***$P < 0.001$ compared with agonist-stimulated control.

examined in additional cell types as shown in Fig. 2. We utilised the prostate cancer cell line DU145 (panel A), as a comparator cancer cell line and a primary cultured cell type, human coronary artery endothelial cells (HCAECs) (panel B). We found in both cell types the same rapid increase in p100 phosphorylation; for DU145 a peak was reached at 30 min (Fold increase: IL-1β, 30 min = 18.98 ± 3.3, ***$P < 0.001$) whilst for HCAECs the peak was even earlier at 15 min (Fold increase: IL-1β, 15 min = 9.73 ± 1.9, **$P < 0.01$). As in agreement with the U2OS cells, IL-1β did not induce any cellular change of p52, nor p100, in either cell type. These results indicate that p100 phosphorylation induced by IL-1β is not just a feature of bone cancer cells but is apparent in multiple cell types.

As activation of the non-canonical NFκB pathway and p100 phosphorylation by non-canonical ligands is recognised to be regulated by IKKα, we sought to determine if the increase in p100 phosphorylation, in response to IL-1β, was regulated in the same manner. We therefore developed an IKKα CRISPR/Cas9 deletion U2OS cell line (Fig. 3). Whilst the non-targeting vector was without effect and gave the same robust response to IL-1β as shown in wild type cells, cellular deletion of IKKα significantly reduced IL-1β phosphorylation of p100 by over 80% at all-time points tested (Panel A). Panel B shows that IKKα deletion also significantly reduced LTα$_1$β$_2$-stimulated phosphorylation recorded at longer time points from 6 to 24 h, again with over 80% inhibition. In addition, as an increase in cellular p52 formation is also a feature of LTα1β2 activation, samples were re-probed for p100/p52. IKKα deletion also inhibited agonist-stimulated p52 accumulation at all time-points. Furthermore, the effect of IKKα deletion was replicated using TNFα as the activating ligand over a similar time course (Panel C), with over 80% inhibition throughout.

In addition to serine 866 and 870, a key IKKα dependent phosphorylation site within p100 is serine 872 [6]. We therefore examined if IKKα deletion effected IL-1β mediated phosphorylation at this site as shown in Fig. 4. For both IL-1β and LTα$_1$β$_2$ we found a similar dependence on IKKα, in wild type and non-targeting (N/T) cells there was a robust response to both agents, in deleted cells the response to both agents was significantly reduced by over each 80% at each time point assessed. This confirms the requirement for IKKα.

Our attempts to develop an IKKβ CRISPR deletion U2OS cells line were not successful. We therefore used siRNA to determine if IKKβ has a role in regulating IL-1β-induced p100 phosphorylation. Knockdown of both IKKα and IKKα by siRNA was initially employed as shown in Fig. 5. Whilst non-targeted siRNA (N/T) was without effect, relative to WT cells, IKKα siRNA as expected significantly reduced IL-1β induced p100 phosphorylation by over 70% (Fold increase: IL-1β + NT = 7.85 ± 0.86 vs. siRNA IKKα + IL-1β = 1.94 ± 0.28, ****$P < 0.0001$) (Panel A). Knockdown of IKKβ did not significantly affect p100 phosphorylation, although there was a minor reduction observed over a number of experiments at the higher concentrations. To confirm these effects we also used the IKKβ selective inhibitor, IKK2-X1 [27]. Pre-treatment of U2OS cells with this compound was ineffective against IL-1β induced p100 phosphorylation at concentrations of up to 30 μM (Panel B) (Fold increase: IL-1β + DMSO = 6.90 ± 1.3 vs. IL-1β + IKK2-X1(30 μM) = 5.47 ± 0.29, ns). However, this inhibitor was effective when tested against IL-1β induced cellular loss of IκBα and phosphorylation of p65 (panel C), confirming its activity against IKKβ. This further suggests that IKKβ does not play a role and further supports selective involvement of IKKα in the regulation of phosphorylation of p100.

The role of NIK in IL-1β mediated p100 phosphorylation was also examined in Fig. 6 and compared to LTα$_1$β$_2$ stimulation. Pre-treatment with increasing concentrations of a recently characterised NIK inhibitor, CW15337 [22] had no effect on IL-1β induced phosphorylation even up to concentrations as high as 20 μM (Panel A). By contrast the response to LTα$_1$β$_2$ was extremely susceptible to the inhibitor, a significant reduction in p100 phosphorylation was observed at concentrations as low as 1 μM (Fold increase: LTα$_1$β$_2$ + DMSO = 5.16 ± 1.08 vs. LTα$_1$β$_2$ + CW 20 μM = 2.06 ± 0.4, *$P < 0.05$) (Panel B).

We also examined the characteristics of nuclear translocation of p52 NFκB in U2OS cells. Initially we examined the time courses of both LTα$_1$β$_2$ and IL-1β, previously outlined in Fig. 1. LTα$_1$β$_2$ -induced p52 induction was typically delayed, reaching a peak by 4–6 h and being sustained for up to 24 h. In contrast IL-1β stimulated a significant increase in nuclear p52 peaking at 60 min, which was sustained for up to 4 h. In further experiments, shown in Figs. 7 & 8 we assessed the relative involvement of IKKα and NIK.

As shown in Fig. 7, Panel A we found that IKKα deletion had a significant effect upon IL-1β -stimulated p52 translocation at 1- and 2-h time points reducing the response by approximately 40% (Fold increase (1 h): N/T + IL-1 = 2.2 ± 0.177 vs. IKKα KD + IL-1 = 1.42 ± 0.057, P > **0.01). In addition, we observed a similar trend with IL-1β -stimulated p65 translocation, however this decrease was not significant (Fold increase (2 h): N/T + IL-1 = 3.10 ± 0.76 vs. IKKα KD + IL-1 = 2.21 ± 0.42). Moreover, following IKKα deletion, the response to LTα$_1$β$_2$ was essentially abolished at two different time points, 4 and 6 h respectively (Fold increase (4 h) N/T + LTX = 1.9 ± 0.18 vs. IKKα KD + LTX = 1.07 ± 0.13, *$P < 0.05$) (Panel B).

Further experiments examined the role of NIK in IL-1β mediated translocation of p52 to the nucleus, as show in Fig. 8. Treatment with IL-1β gave an approximately 3-fold stimulation of p52 accumulation (Fold Stimulation: IL-1 + DMSO = 3.01 ± 0.42, *P < 0.05), however pre-incubation of cells with CW15337 had no effect on this stimulation (Panel A). In contrast the response to LTα$_1$β$_2$ was again very sensitive to the compound (Panel B); the response was essentially abolished at both

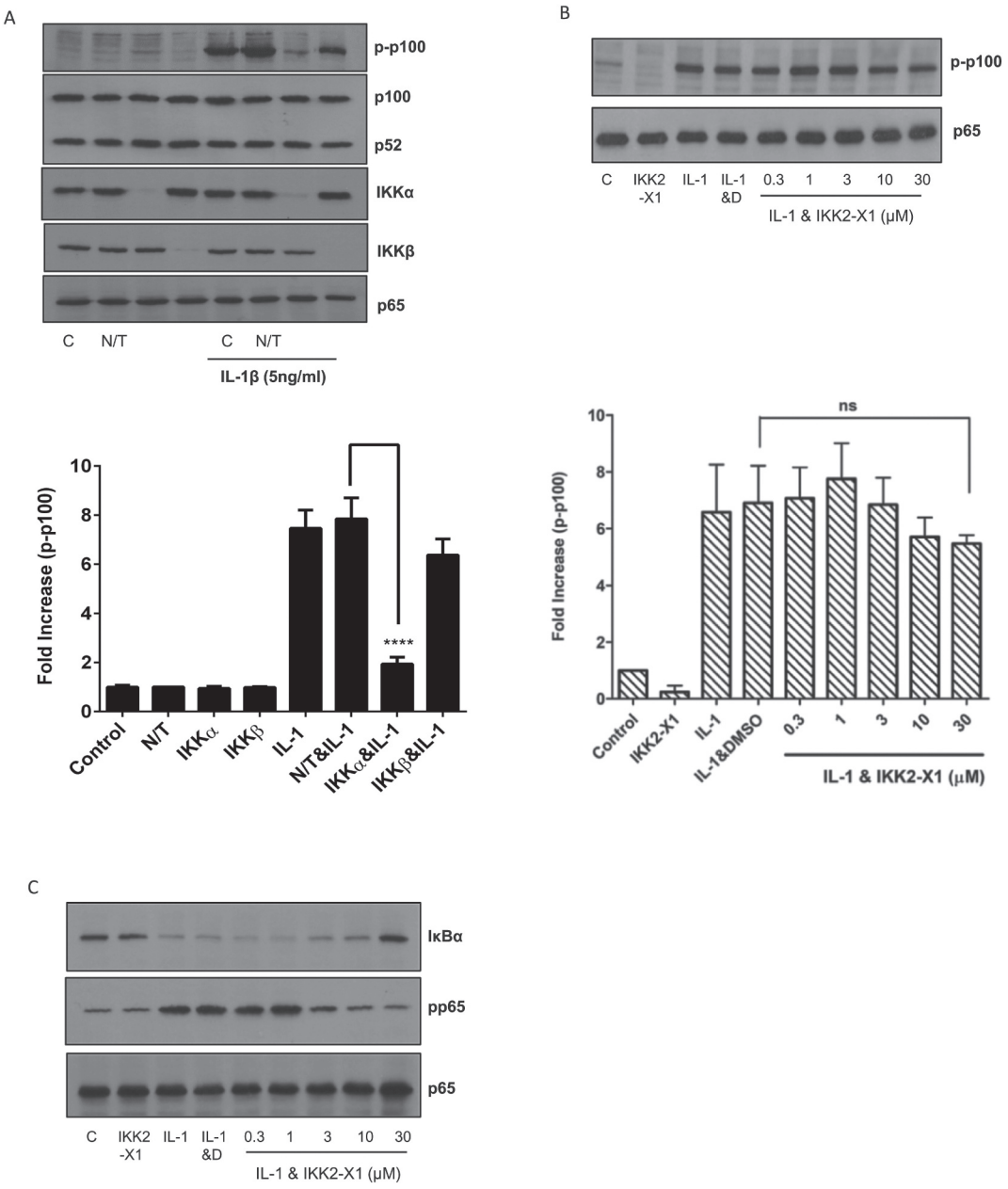


Fig. 5. The effect of siRNA IKKα and β or IKKβ inhibitor IKK2-XI on IL-1β stimulated phosphorylation of p100 in U2OS cells.
U2OS cells were transfected with non-targeting siRNA, siRNA IKKα or siRNA IKKβ (50 nM) for 72 h prior to stimulation with IL-1β (5 ng/ml) for a further 30 min (Panel A). U2OS cells were pre-incubated with increasing concentrations of IKK2-XI for 30 min prior to stimulation with IL-1β (5 ng/ml) for 30 min (Panel B & C). Whole cell lysates were assessed for, p-p100, p100/p52, IKKα, IKKβ, IκBα, pp65 and p65 used as loading control. Blots were semi-quantified using densitometry; results are representative of at least three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with agonist-stimulated control.

1 and 10 μM (Fold increase: LTX + DMSO = 3.44 ± 0.65 vs. LTX + CW-10 μM = 1.5 ± 0.14-fold, $^{*}P > 0.05$), confirming the dependency of the $LT\alpha_1\beta_2$ to NIK.

5. Discussion

In this paper, we have identified for the first time a rapid IKKα -dependent, NIK -independent activation of p100 phosphorylation in response to ligands that activate the canonical NFκB pathway strongly namely, IL-1β and TNFα. This suggests a hitherto unidentified input into the non-canonical NFκB pathway to that previously explored.

Initially, we found a delayed, slow phosphorylation of p100 from 4 h in response to the non-canonical NFκB ligand, $LT\alpha_1\beta_2$. Whilst this is a well-recognised event in activation of non-canonical signalling [5,6], there is a significant paucity in the literature regarding measurement of agonist stimulated p100 phosphorylation in cells, and very few studies show this phenomenon. However the kinetics of activation by $LT\alpha_1\beta_2$ are consistent with a number of studies in other cells types such as endothelial cells [28,29], stimulated by LIGHT ligand or $LT\alpha_1\beta_2$ or other cell lines [30], where activation of p52 formation is a marker of non-canonical NFκB pathway activation. Indeed, $LT\alpha_1\beta_2$ mediated p100 phosphorylation precedes or is co-incident with cellular p52 formation and accumulation within the nucleus (Fig. 1), starting at approximately 4 h. This suggests that in U2OS cells p100 phosphorylation is indicative of non-canonical NFκB pathway activation in response to the cognate ligand, $LT\alpha_1\beta_2$ and proceeds in the normal manner in this cell line.

In stark contrast, we identified a rapid increase in the phosphorylation of p100 in response to either IL-1β or TNFα in a number of different

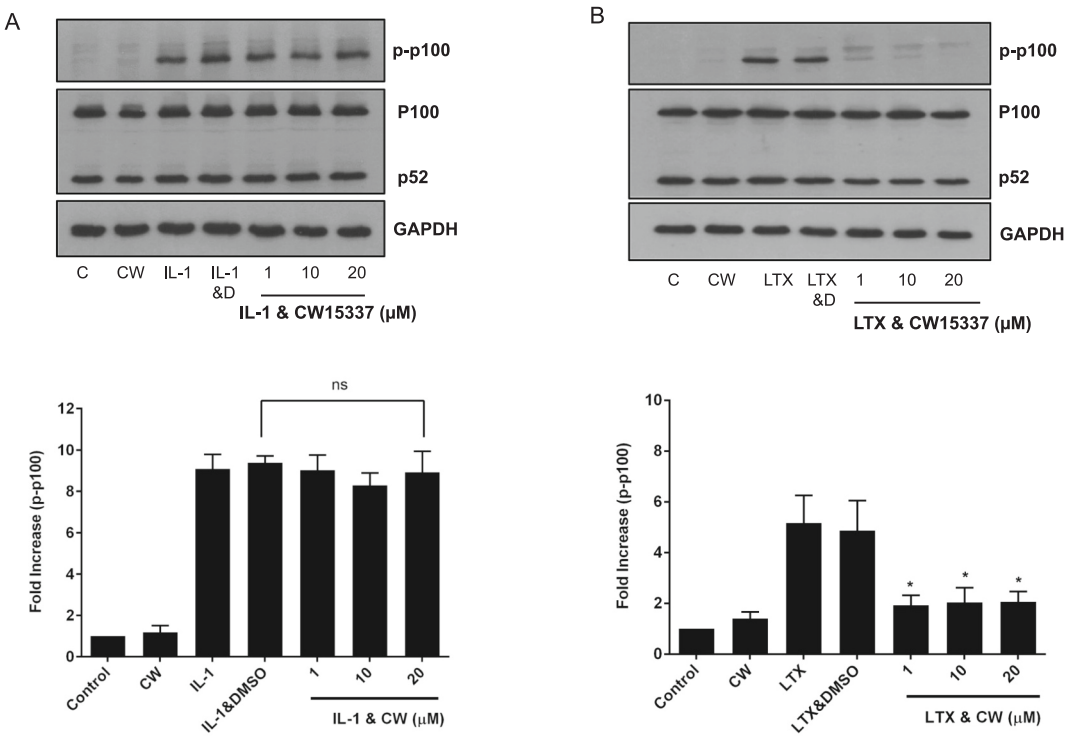


Fig. 6. Effect of the NIK inhibitor CW15337 on IL-1β and LTα1β2 induced p100 phosphorylation U2OS cells.
U2OS cells were pre-treated with CW15337 for 30 min prior to stimulation with IL-1β (5 ng/ml) for 30 min (Panel A) or $LT\alpha_1\beta_2$ (20 ng/ml) for 4 h (Panel B). Whole cell lysates were assessed for, p-p100, p100/p52, and GAPDH used as loading control. Blots were semi-quantified using densitometry; results are representative of at least three independent experiments. $^*P < 0.05$, compared with agonist-stimulated control.

cell types suggesting a ubiquitous phenomenon. This finding goes against the current dogma that ligands that drive the canonical NFκB pathway do not activate the non-canonical NFκB pathway, based primarily on the assessment of cellular p52 formation. A number of studies have indicated the potential for TNFα, through TNFR2, to stimulate the non-canonical NFκB pathway as measured by p52 formation; however, this again is usually following prolonged (18 h) stimulation [31]. TNFα induced p52 formation is also found when TRAF2 signalling is disrupted by either TRAF2 or RIP deletion [32,33], or in cells deficient in p100 [34] suggesting the potential of activation of the non-canonical NFκB axis in response to canonical ligands under certain experimental conditions. However, p100 phosphorylation was not assessed in any of these studies to confirm these findings nor was early kinetics of stimulation examined. In our hands, the canonical NFκB ligand TNFα consistently gave a rapid activation in wild type cells in which elements upstream or downstream have not been altered/deleted suggesting a different mode of activation.

Given that our findings indicate a novel, kinetically distinct activation of the pathway, it was important to determine if IKKα was involved. This was achieved using molecular approaches. Our studies using a CRISPR/Cas9 IKKα deletion cell line confirmed an IKKα -mediated activation for both IL-1β and TNFα and, as a control, $LT\alpha_1\beta_2$, which again demonstrates that IKKα is integral to control of the non-canonical NFκB pathway in these cells. This is consistent with previous studies that show loss in IKKα prevented $LT\alpha_1\beta_2$ or LIGHT induced p52 formation [5,28,29]. However, given that IKKα is essential for p100 phosphorylation, this suggests that both IL-1β and TNFα receptors must share a common pathway for activation. Further molecular and pharmacological experiments excluded a role for IKKβ which would be consistent with early studies that showed IKKβ, at least in vitro, was unable to significantly phosphorylate p100 [5]. Other common pathways mediated by ligands IL-1β and TNFα include the MAP kinases, although little evidence supports a role for these kinases in this pathway. A number of studies have implicated AKT in either the phosphorylation of IKKα [35] or regulation of non-canonical NFκB signalling through LPA-liganded receptors [36] and this mechanism may be involved here.

A significant additional finding was the lack of susceptibility of IL-1β -mediated p100 phosphorylation to NIK inhibition, which contrasted with the response to $LT\alpha_1\beta_2$. $LT\alpha_1\beta_2$. Stimulation was extremely sensitive to the inhibitor clearly confirming a role for NIK in the coupling of $LT\alpha_1\beta_2$ to the non-canonical pathway. Indeed, CW15337 has recently been shown to strongly inhibit the activation of non-canonical NFκB signalling [22] suggesting it is an appropriate pharmacological tool to interrogate the pathway. Previous studies have also demonstrated an essential role for NIK in mediating not only the activation of IKKα but also in promoting the interaction of IKKα with p100 [5–7]. This suggests that for IL-1β stimulation, NIK is not required for the association of IKKα with p100 to induce phosphorylation. As judged by the rapid phosphorylation kinetics in response to IL-1β, there is unlikely to be sufficient time for NIK to be induced. This suggests that another binding partner or intermediate may play a role in bringing IKKα into a complex with p100 in response to IL-1β stimulation. As indicated above this could be part of a common pathway regulated by both IL-1β and TNFα and as NIK is a MAP3kinase it may be another family member such as the MEKKs and TAK-1 which is integral to the IL-1β signalling cascade [37–39]. However, irrespective of the mechanism involved, the interaction between IKKα and p100 mediated by IL-1β is weaker or at least transient, relative to the sustained interaction normally driven by NIK, suggesting a different regulatory mode within the pathway.

Furthermore, in this study we found that IL-1β stimulated a rapid increase in the nuclear translocation of p52, which was partially dependent on IKKα. Such a paradigm, has not been extensively explored to date with respect to p52; the current model suggests that non-canonical signalling results in the NIK induced processing of p100 such that p52 once formed will translocate to the nucleus usually as a p52/RelB dimer [40]. This is in part due to the presence of an NLS in the

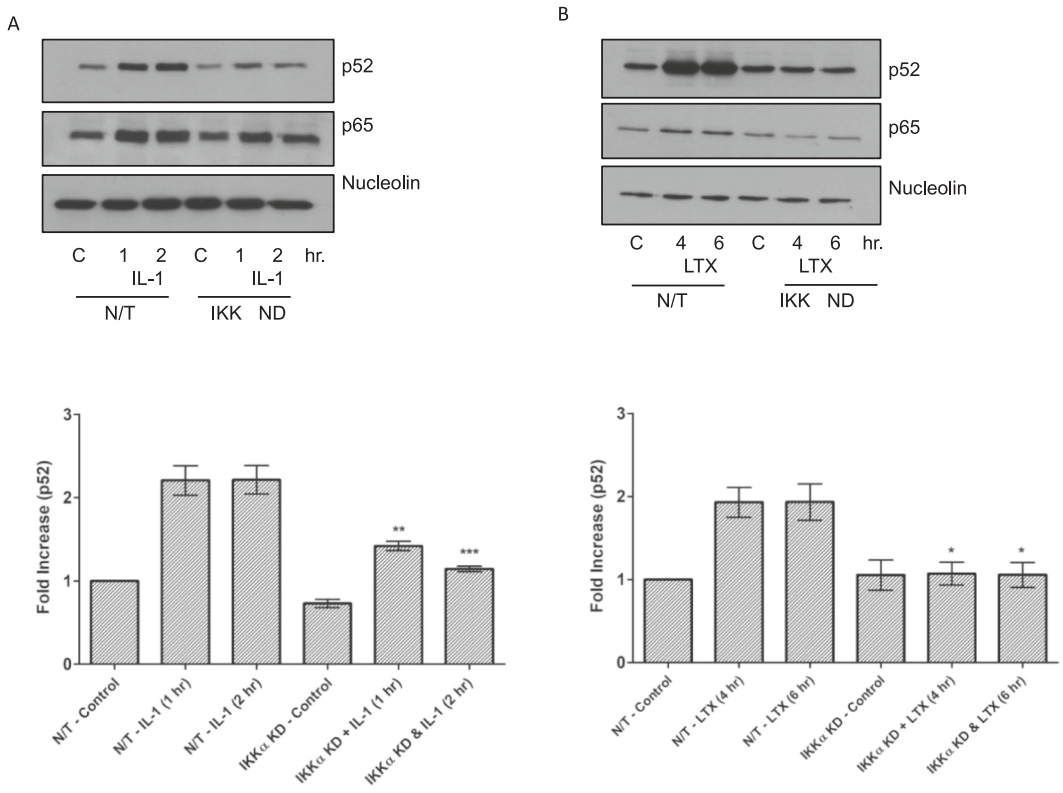


Fig. 7. Effect of IKKα deletion on IL-1β or LTα1β2 - induced nuclear p52 NFκB translocation in U2OS cells.
Cas 9 (N/T) or IKKα CRISPR/Cas9 U2OS cells were incubated with IL-1β (5 ng/ml) (Panel A) or $LT\alpha_1\beta_2$ (20 ng/ml) (Panel B) for the times indicated. Nuclear extracts were assessed for p52, p65 and nucleolin used as loading control. Blots were semi-quantified using densitometry; results are representative of at least three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}P < 0.001$ compared with agonist-stimulated control.

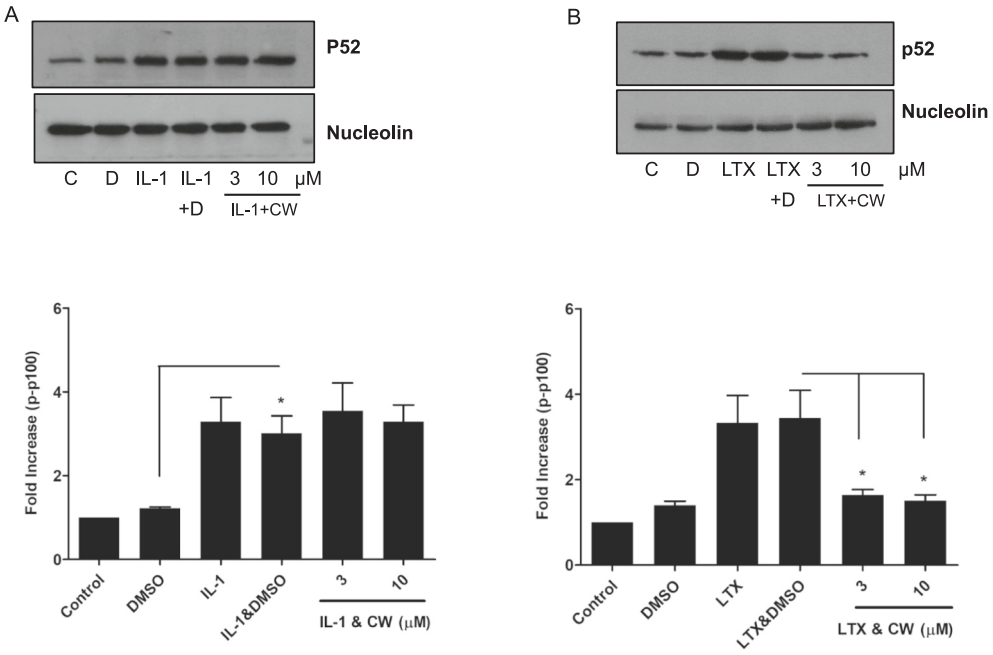


Fig. 8. CW15337 reduces $LT\alpha_1\beta_2$ but not IL-1β- induced p52 NFκB nuclear translocation in U2OS cells.
U2OS cells were pre-treated with CW15337 for 30 min prior to stimulation with IL-1β (5 ng/ml) for 30 min (Panel A) or LTα1β2 (20 ng/ml) for 4 h (Panel B). Nuclear extracts were assessed for p100/p52, and nucleolin used as loading control. Blots were semi-quantified using densitometry; the results are representative of at least three independent experiments. $^*P < 0.05$ compared with agonist-stimulated control.

C-terminus of p52 which allows interactions with specific importins [41]. Our results suggest that the source of p52 is from the cytosol and does not involve p100 processing, which makes the pathway distinct from the normal non-canonical NFκB cascade.

The regulation of p52 has been studied extensively by Ghosh and co-workers [40]; a number of studies have indicated the presence of a multimeric p100 complex in the cytosol as part of a NFκBsome [42] which functions as a constraining IκB which is able to sequester a number of NFκB subunits including p52 [43]. Such a pool is thought to be distinct from the p100 pool involved in processing [44]. Thus, IL-1β

through the phosphorylation of this pool of p100 may be able to mediate the dissociation of p52 and promote nuclear translocation in the absence of RelB for example as part of a p52/RelA dimer. However, a more detail kinetic analysis of the relative kinetics of p52 and RelB nuclear translocation maybe be required to determine if sufficient p52 is liberated in the absence of RelB. Recently, it has been shown by Cook and co-workers that the deletion of IKKα reduces p65 translocation to the nucleus [45], a result replicated in the current study. It is possible, therefore, that p65 may translocate to the nucleus as a RelA/p52 dimer and the reduction in translocation of p65 concomitantly reduces the translocation of p52.

In conclusion, we have identified a novel input into the non-canonical NFκB pathway by canonical ligands, a departure from the normal paradigm for activation. How this regulates physiological outcomes within the cell remains to be established.

Credit author statement

Kathryn McIntosh: Conceptualization, Methodology, Data curation, Data analysis, preparation of figures, manuscript review/writing. **Robin Plevin.**: Supervision, Conceptualization Data curation, Writing- Original draft preparation. **Yousif Khalaf**: Data curation, **Rachel Craig**: Data curation, Data analysis**: Christopher West**: Compound generation: **Ashley McCulloch:** Data curation, **Ajay Wagmare**: Methodology, Data Curation, **Christopher Lawson**; Compound generation, **Edmond Chan**: Methodology, Generation of CRISPR KO lines, **Simon MacKay**: Manuscript review, **Andrew Paul**: manuscript review.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by studentships from the Ministry of Higher Education and Scientific Research, Iraq, (Y·K) and the Maharashtra State Government, India, (A.W).

References

[1] J.A. Prescott, J.P. Mitchell, S.J. Cook, Inhibitory feedback control of NF-kappaB signalling in health and disease, Biochem. J. 478 (2021) 2619–2664.
[2] C. Gamble, K. McIntosh, R. Scott, K.H. Ho, R. Plevin, A. Paul, Inhibitory kappa B kinases as targets for pharmacological regulation, Br. J. Pharmacol. 165 (2012) 802–819.
[3] S.C. Sun, The non-canonical NF-kappaB pathway in immunity and inflammation, Nat. Rev. Immunol. 17 (2017) 545–558.
[4] C. Dostert, M. Grusdat, E. Letellier, D. Brenner, The TNF family of ligands and receptors: communication modules in the immune system and beyond, Physiol. Rev. 99 (2019) 115–160.
[5] C. Liang, M. Zhang, S.C. Sun, Beta-TrCP binding and processing of NF-kappaB2/p100 involve its phosphorylation at serines 866 and 870, Cell. Signal. 18 (2006) 1309–1317.
[6] G. Xiao, A. Fong, S.C. Sun, Induction of p100 processing by NF-kappaB-inducing kinase involves docking IkappaB kinase alpha (IKKalpha) to p100 and IKKalpha-mediated phosphorylation, J. Biol. Chem. 279 (2004) 30099–30105.
[7] G. Xiao, E.W. Harhaj, S.C. Sun, NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100, Mol. Cell 7 (2001) 401–409.
[8] G. Liao, M. Zhang, E.W. Harhaj, S.C. Sun, Regulation of the NF-kappaB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation, J. Biol. Chem. 279 (2004) 26243–26250.
[9] S. Vallabhapurapu, A. Matsuzawa, W. Zhang, P.H. Tseng, J.J. Keats, H. Wang, D. A. Vignali, P.L. Bergsagel, M. Karin, Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling, Nat. Immunol. 9 (2008) 1364–1370.
[10] B.J. Zarnegar, Y. Wang, D.J. Mahoney, P.W. Dempsey, H.H. Cheung, J. He, T. Shiba, X. Yang, W.C. Yeh, T.W. Mak, R.G. Korneluk, G. Cheng, Noncanonical NF-kappaB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK, Nat. Immunol. 9 (2008) 1371–1378.
[11] D.V. Novack, L. Yin, A. Hagen-Stapleton, R.D. Schreiber, D.V. Goeddel, F.P. Ross, S. L. Teitelbaum, The IkappaB function of NF-kappaB2 p100 controls stimulated osteoclastogenesis, J. Exp. Med. 198 (2003) 771–781.
[12] S. Vaira, M. Alhawagri, I. Anwisye, H. Kitaura, R. Faccio, D.V. Novack, RelA/p65 promotes osteoclast differentiation by blocking a RANKL-induced apoptotic JNK pathway in mice, J. Clin. Invest. 118 (2008) 2088–2097.
[13] K.L. Culley, S.G. Lessard, J.D. Green, J. Quinn, J. Chang, T. Khilnani, E. B. Wondimu, C.L. Dragomir, K.B. Marcu, M.B. Goldring, M. Otero, Inducible knockout of CHUK/IKKalpha in adult chondrocytes reduces progression of cartilage degradation in a surgical model of osteoarthritis, Sci. Rep. 9 (2019) 8905.
[14] I. Holen, D.V. Lefley, S.E. Francis, S. Rennicks, S. Bradbury, R.E. Coleman, P. Ottewell, IL-1 drives breast cancer growth and bone metastasis in vivo, Oncotarget 7 (2016) 75571–75584.
[15] Y. Tong, Y. Cao, T. Jin, Z. Huang, Q. He, M. Mao, Role of Interleukin-1 family in bone metastasis of prostate cancer, Front. Oncol. 12 (2022), 951167.
[16] S. Yati, A. Silathapanasakul, C. Thakaeng, M. Chanasakulniyom, N. Songtawee, S. Porntadavity, P. Pothacharoen, D. Pruksakorn, P. Kongtawelert, P. T. Yenchitsomanus, T. Chanmee, Extracellular vesicle-mediated IL-1 Signaling in response to doxorubicin activates PD-L1 expression in osteosarcoma models, Cells 11 (2022).
[17] B.F. Boyce, T.B. Aufdemorte, I.R. Garrett, A.J. Yates, G.R. Mundy, Effects of interleukin-1 on bone turnover in normal mice, Endocrinology 125 (1989) 1142–1150.
[18] P. Seckinger, J. Klein-Nulend, C. Alander, R.C. Thompson, J.M. Dayer, L.G. Raisz, Natural and recombinant human IL-1 receptor antagonists block the effects of IL-1 on bone resorption and prostaglandin production, J. Immunol. 145 (1990) 4181–4184.
[19] L. Xing, L. Carlson, B. Story, Z. Tai, P. Keng, U. Siebenlist, B.F. Boyce, Expression of either NF-kappaB p50 or p52 in osteoclast precursors is required for IL-1-induced bone resorption, J. Bone Miner. Res. 18 (2003) 260–269.
[20] N.G. Anthony, J. Baiget, G. Berretta, M. Boyd, D. Breen, J. Edwards, C. Gamble, A. I. Gray, A.L. Harvey, S. Hatziieremia, K.H. Ho, J.K. Huggan, S. Lang, S. Llona-Minguez, J.L. Luo, K. McIntosh, A. Paul, R.J. Plevin, M.N. Robertson, R. Scott, C. J. Suckling, O.B. Sutcliffe, L.C. Young, S.P. Mackay, Inhibitory kappa B kinase alpha (IKKalpha) inhibitors that recapitulate their selectivity in cells against isoform-related biomarkers, J. Med. Chem. 60 (2017) 7043–7066.
[21] C. Garcia-Moruja, J.M. Alonso-Lobo, P. Rueda, C. Torres, N. Gonzalez, M. Bermejo, F. Luque, F. Arenzana-Seisdedos, J. Alcami, A. Caruz, Functional characterization of SDF-1 proximal promoter, J. Mol. Biol. 348 (2005) 43–62.
[22] M. Haselager, R. Thijssen, C. West, L. Young, R. Van Kampen, E. Willmore, S. Mackay, A. Kater, E. Eldering, Regulation of Bcl-XL by non-canonical NF-kappaB in the context of CD40-induced drug resistance in CLL, Cell Death Differ. 28 (2021) 1658–1668.
[23] N.E. Sanjana, O. Shalem, F. Zhang, Improved vectors and genome-wide libraries for CRISPR screening, Nat. Methods 11 (2014) 783–784.
[24] J.G. Doench, N. Fusi, M. Sullender, M. Hegde, E.W. Vaimberg, K.F. Donovan, I. Smith, Z. Tothova, C. Wilen, R. Orchard, H.W. Virgin, J. Listgarten, D.E. Root, Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9, Nat. Biotechnol. 34 (2016) 184–191.
[25] C.J. MacKenzie, E. Ritchie, A. Paul, R. Plevin, IKKalpha and IKKbeta function in TNFalpha-stimulated adhesion molecule expression in human aortic smooth muscle cells, Cell. Signal. 19 (2007) 75–80.
[26] G.D. Bren, N.J. Solan, H. Miyoshi, K.N. Pennington, L.J. Pobst, C.V. Paya, Transcription of the RelB gene is regulated by NF-kappaB, Oncogene 20 (2001) 7722–7733.
[27] A. Baxter, S. Brough, A. Cooper, E. Floettmann, S. Foster, C. Harding, J. Kettle, T. McInally, C. Martin, M. Mobbs, M. Needham, P. Newham, S. Paine, S. St-Gallay, S. Salter, J. Unitt, Y. Xue, Hit-to-lead studies: the discovery of potent, orally active, thiophenecarboxamide IKK-2 inhibitors, Bioorg. Med. Chem. Lett. 14 (2004) 2817–2822.
[28] P. Kucharzewska, C.X. Maracle, K.C.M. Jeucken, J.P. van Hamburg, E. Israelsson, M. Furber, S.W. Tas, H.K. Olsson, NIK-IKK complex interaction controls NF-kappaB-dependent inflammatory activation of endothelium in response to LTbetaR ligation, J. Cell Sci. 132 (2019).
[29] L.A. Madge, M.S. Kluger, J.S. Orange, M.J. May, Lymphotoxin-alpha 1 beta 2 and LIGHT induce classical and noncanonical NF-kappa B-dependent proinflammatory gene expression in vascular endothelial cells, J. Immunol. 180 (2008) 3467–3477.
[30] B. Mordmuller, D. Krappmann, M. Esen, E. Wegener, C. Scheidereit, Lymphotoxin and lipopolysaccharide induce NF-kappaB-p52 generation by a co-translational mechanism, EMBO Rep. 4 (2003) 82–87.
[31] H. Rauert, A. Wicovsky, N. Muller, D. Siegmund, V. Spindler, J. Waschke, C. Kneitz, H. Wajant, Membrane tumor necrosis factor (TNF) induces p100 processing via TNF receptor-2 (TNFR2), J. Biol. Chem. 285 (2010) 7394–7404.
[32] J.Y. Kim, M. Morgan, D.G. Kim, J.Y. Lee, L. Bai, Y. Lin, Z.G. Liu, Y.S. Kim, TNFalpha induced noncanonical NF-kappaB activation is attenuated by RIP1 through stabilization of TRAF2, J. Cell Sci. 124 (2011) 647–656.
[33] J. Liu, T. Hideshima, L. Xing, S. Wang, W. Zhou, M.K. Samur, T. Sewastianik, D. Ogiya, G. An, S. Gao, L. Yang, T. Ji, G. Bianchi, K. Wen, Y.T. Tai, N. Munshi, P. Richardson, R. Carrasco, Y. Cang, K.C. Anderson, ERK signaling mediates resistance to immunomodulatory drugs in the bone marrow microenvironment, Sci. Adv. 7 (2021).

[34] A. Lovas, A. Weidemann, D. Albrecht, L. Wiechert, D. Weih, F. Weih, p100 deficiency is insufficient for full activation of the alternative NF-kappaB pathway: TNF cooperates with p52-RelB in target gene transcription, PLoS One 7 (2012), e42741.
[35] D. Bai, L. Ueno, P.K. Vogt, Akt-mediated regulation of NFkappaB and the essentialness of NFkappaB for the oncogenicity of PI3K and Akt, Int. J. Cancer 125 (2009) 2863–2870.
[36] G. Mao, S.S. Smyth, A.J. Morris, Regulation of PLPP3 gene expression by NF-kappaB family transcription factors, J. Biol. Chem. 294 (2019) 14009–14019.
[37] J. Totzke, D. Gurbani, R. Raphemot, P.F. Hughes, K. Bodoor, D.A. Carlson, D. R. Loiselle, A.K. Bera, L.S. Eibschutz, M.M. Perkins, A.L. Eubanks, P.L. Campbell, D. A. Fox, K.D. Westover, T.A.J. Haystead, E.R. Derbyshire, Takinib, a selective TAK1 inhibitor, broadens the therapeutic efficacy of TNF-alpha inhibition for cancer and autoimmune disease, Cell Chem. Biol. 24 (1029–1039) (2017), e1027.
[38] Z.Y. Zhang, S.Z. Li, H.H. Zhang, Q.R. Wu, J. Gong, T. Liang, L. Gao, N.N. Xing, W. B. Liu, R.L. Du, X.D. Zhang, Stabilization of ATF5 by TAK1-Nemo-like kinase critically regulates the interleukin-1beta-stimulated C/EBP signaling pathway, Mol. Cell. Biol. 35 (2015) 778–788.
[39] J. Ninomiya-Tsuji, K. Kishimoto, A. Hiyama, J. Inoue, Z. Cao, K. Matsumoto, The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway, Nature 398 (1999) 252–256.
[40] G. Ghosh, V.Y. Wang, Origin of the functional distinctiveness of NF-kappaB/p52, Front. Cell Dev. Biol. 9 (2021), 764164.
[41] R. Fagerlund, K. Melen, X. Cao, I. Julkunen, NF-kappaB p52, RelB and c-Rel are transported into the nucleus via a subset of importin alpha molecules, Cell. Signal. 20 (2008) 1442–1451.
[42] Z. Tao, A. Fusco, D.B. Huang, K. Gupta, D. Young Kim, C.F. Ware, G.D. Van Duyne, G. Ghosh, p100/IkappaBdelta sequesters and inhibits NF-kappaB through kappaBsome formation, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 15946–15951.
[43] S. Basak, V.F. Shih, A. Hoffmann, Generation and activation of multiple dimeric transcription factors within the NF-kappaB signaling system, Mol. Cell. Biol. 28 (2008) 3139–3150.
[44] Z.B. Yilmaz, B. Kofahl, P. Beaudette, K. Baum, I. Ipenberg, F. Weih, J. Wolf, G. Dittmar, C. Scheidereit, Quantitative dissection and modeling of the NF-kappaB p100-p105 module reveals interdependent precursor proteolysis, Cell Rep. 9 (2014) 1756–1769.
[45] J.A. Prescott, K. Balmanno, J.P. Mitchell, H. Okkenhaug, S.J. Cook, IKKalpha plays a major role in canonical NF-kappaB signalling in colorectal cells, Biochem. J. 479 (2022) 305–325.